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# Proposed mechanism of antibacterial mode of action of *Caesalpinia bonducella* seed oil against food-borne pathogens

**Shruti Shukla[1], Rajib Majumder[2], Laxmi Ahirwal[3] and Archana Mehta[3]**

*[1]Department of Food Science and Technology, Yeungnam University, 280 Daehak-Ro, Gyeongsan-Si, Gyeongsangbuk-Do 712 749, Republic of Korea; [2]Department of Applied Microbiology and Biotechnology, Yeungnam University, Gyeongsan, Gyeongbuk 712 749, Republic of Korea; [3]Laboratory of Plant Pathology and Microbiology, Department of Botany, Dr. Hari Singh Gour University, Sagar 470 003 MP, India.*

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## Abstract

The antibacterial mechanism of action of *Caesalpinia bonducella* seed oil on membrane permeability of *Listeria monocytogenes* NCIM 24563 (MIC: 2 mg/mL) and *Escherichia coli* ATCC 25922 (MIC: 4 mg/mL) was determined by measuring the extracellular ATP concentration, release of 260-nm absorbing materials, leakage of potassium ions and measurement of relative electrical conductivity of the bacterial cells treated at MIC concentration. Its mode of action on membrane integrity was confirmed by release of extracellular ATP (1.42 and 1.33 pg/mL), loss of 260-nm absorbing materials (4.36 and 4.19 optical density), leakage of potassium ions (950 and 1000 mmol/L) and increase in relative electrical conductivity (12.6 and 10.5%) against food-borne pathogenic bacteria *L. monocytogenes* and *E. coli*, respectively. These findings propose that *C. bonducella* oil compromised its mode of action on membrane integrity, suggesting its enormous food and pharmacological potential.

## Introduction

Food-borne disease or food poisoning is any illness resulting from the consumption of food contaminated with food-borne pathogenic bacteria, which has been of great concern to public health (Tauxe, 2002). Since long, synthetic chemicals have been used to control microbial growth and to reduce the incidence of food-borne illness (Zurenko et al., 1996). Although these synthetic preservatives are effective, they might be detrimental to human health (Aiyelaagbe et al., 2007). Hence, there is a huge attention on plant-based novel antibacterial agents such as essential oils for controlling food-borne pathogens as safe and natural remedies (Giang et al., 2006). The application of essential oils as safe and effective alternatives to synthetic preservatives in controlling pathogens may possibly able to reduce the risk of food-borne outbreaks and may assure consumers with safe food products (Yoon et al., 2011).

*Caesalpinia bonducella* (L.) Roxb. is often grown as hedge plant, and found throughout the hotter parts of India. *C. bonducella* seeds are used by the tribal people in India for controlling blood sugar and for the treatment of diabetes mellitus, asthma and chronic fever (Chakrabarti et al., 2003). All parts of *C. bonducella* (leaf, root and seed) are used in traditional system of medi-cines. Previously we reported the antibacterial, antidia-betic, antioxidant, anti-inflammatory, anti-pyretic and analgesic properties of *C. bonducella* seed oil (Shukla et al., 2010).

Previous results on the antibacterial screening of *C. bonducella* seed oil showed that it had strong and consistent inhibitory effect against various food-borne pathogens (Shukla et al., 2013). The seed oil displayed varying degree of susceptibility against various food-borne pathogens including *L. monocytogenes* and *E. coli* within a range of 2 to 8 mg/mL (Shukla et al., 2013).

Present study evaluated the antibacterial effect of *C. bonducella* seed oil in various *in vitro* assays against selected food-borne pathogens, and proposed its possible antibacterial mode of action.

## Materials and Methods

### *Collection of sample and oil preparation*

The seeds of *C. bonducella* were collected in March 2006 from Sagar District, Madhaya Pradesh, India and further taxonomic identification was conducted by Herbarium In-charge, Department of Botany, Dr. H. S. Gour University, Sagar, MP, India. A voucher specimen has been deposited in the Herbarium of Laboratory of Botany Department, under the number (Bot/H/2692). The seeds were dried, powdered and passed through 40 -mesh sieve and stored in an airtight container for further use (Shukla et al., 2010). The powdered seed material (100 g) was subjected to hydrodistillation for 3 hours, using a Clevenger apparatus. The oil was dried over anhydrous $Na_2SO_4$ and preserved in an airtight vial at low temperature (4°C) for further analysis (Shukla et al., 2010).

### *Microorganisms*

The test food-borne pathogens included *Listeria monocytogenes* NCIM 24563 and *Escherichia coli* ATCC 25922. All the bacterial strains were grown in the nutrient broth at 37°C. The bacterial strains were maintained on nutrient agar slants at 4°C.

### *Measurement of extracellular adenosine 5'-triphophate (ATP) concentration*

To determine the efficacy of *C. bonducella* seed oil on membrane integrity, the extracellular ATP concentrations were measured according to the previously described method (Bajpai et al., 2013). The working cultures of *L. monocytogenes* and *E. coli* containing approximately $10^7$ CFU/mL inoculum were centrifuged for 10 min at 1,000 ×g, and the supernatants were removed. The cell pellets were washed three times with 0.1 M of sodium phosphate buffer (pH 7.0) and then cells were collected by centrifugation for 10 min at 1,000 ×g. A cell suspension ($10^7$ CFU/mL) was prepared with 9 mL of sodium phosphate buffer (0.1 M; pH 7.0) and 0.5 mL of cell solution was taken into the Eppendorf tube for the treatment of *C. bonducella* seed oil. Then, the different concentrations (control and MIC) of *C. bonducella* seed oil were added to the cell solution. Samples were maintained at room temperature for 30 min, centrifuged for 5 min at 2,000 ×g, and incubated in ice immediately to prevent ATP loss until measurement. The extracellular (upper layer) ATP concentrations were measured using an ATP bioluminescent assay kit (USA) which comprised ATP assay mix containing luciferase, luciferin, $MgSO_4$, dithiothreitol (DTT), ethylenediamine tetraacetic acid (EDTA), bovine serum albumin and tricine buffer salts. The ATP concentration of the supernatants, which represented the extracellular concentration, was determined using a luminescence reader after the addition of 100 µL of ATP assay mix to 100 µL of supernatant. The emission and excitation wavelengths were 520 and 420 nm, respectively.

### *Assay of potassium ions efflux*

A previously described method was used to determine the amount of the potassium ions (Bajpai et al., 2013). The concentration of free potassium ions in bacterial suspensions of *L. monocytogenes* and *E. coli* was measured after the exposure of bacterial cells to *C. bonducella* seed oil at MIC concentration in sterile peptone water for 0, 30, 60 and 120 min. At each pre-established interval, the extracellular potassium concentration was measured by a photometric procedure using the calcium/potassium kit. Similarly, control was also tested without adding *C. bonducella* seed oil. Results were expressed as amount of extracellular free potassium (mmol/L) in the growth media in each interval of incubation.

### *Measurement of release of 260-nm absorbing cellular materials*

The measurement of the release of 260-nm-absorbing materials from *L. monocytogenes* and *E. coli* cells was carried out in aliquots of 2 mL of the bacterial inocula in sterile peptone water (0.1 g/100 mL) added of *C. bonducella* seed oil MIC concentration at 37°C. At 0, 30 and 60 min time interval of treatment, cells were centrifuged at 3000 xg, and the absorbance of the obtained supernatant was measured at 260 nm using a 96-well plate ELISA reader (Carson et al., 2002). Similarly, control was also tested without adding *C. bonducella* seed oil. Results were expressed in terms of optical density of 260-nm absorbing materials in each interval with respect to the ultimate time.

### *Measurement of cell membrane permeability*

Effect of *C. bonducella* seed oil on cell membrane permeability of test microorganism was determined as described previously (Patra et al., 2015) and expressed in terms of relative electrical conductivity. Prior to the assay, cultures of test microorganisms were incubated at 37°C for 10 hours, followed by centrifugation 4,000 xg for 10 min, and washed with 5% glucose solution (w/v) until their electrical conductivities reached close to 5% glucose solution to induce an isotonic condition. Minimum inhibitory concentrations of *C. bonducella* seed oil acquired for both the test organisms were added to 5% glucose, incubated at 37°C for 8 hours, and the electrical conductivities ($L_a$) of the reaction mixtures were determined. Further, electrical conductivities of the bacterial solutions were measured at 2 hours of

intervals for a total duration of 8 hours ($L_b$). The electrical conductivity of each test pathogen in isotonic solution killed by boiling water for 5 min served as a control ($L_c$). The relative electrical conducti-vity was measured using an electrical conductivity meter. The permeability of bacteria membrane was calculated according to the following formula:

Relative conductivity (%) = $L_a - L_b / L_c \times 100$.

### *Statistical analysis*

All data are expressed as the mean ± SD by measuring three independent replicates. Analysis of variance using one-way ANOVA followed by Duncan's multiple test to test the significance of differences using SPSS software.

## Results

### *Measurement of ATP*

The extracellular ATP concentrations in the control cells of *L. monocytogenes* and *E. coli* were found to be 0.32 and 0.48 pg/mL, respectively (Table I). *L. monocytogenes* and *E. coli* cells treated with *C. bonducella* seed oil at MIC concentration showed significant ($p<0.05$) increase in the release of extracellular ATP concentration. In this assay, the extracellular ATP concentrations for *L. monocytogenes* and *E. coli* cells were measured to be 1.42 and 1.33 pg/mL, respectively (Table I).

**Table I**

**Effect of the *C. bonducella* seed oil on extracellular ATP concentration of *L. monocytogenes* and *E. coli***

| Bacteria | Release of extracellular ATP (pg/mL) | |
|---|---|---|
| | Control | Treatment |
| *L. monocytogenes* NCIM 24563 | 0.32 ± 0.41 | 1.42 ± 0.32 |
| *E. coli* ATCC 25922 | 0.48 ± 0.44 | 1.33 ± 0.46 |

Data are expressed as mean ± SD; (n = 3); Values with different super-scripts are significantly different ($p<0.05$)

### *Measurement of potassium ion leakage*

In this assay, antibacterial mode of action of *C. bonducella* seed oil was confirmed by the release of potassium ions from the treated cells of *L. monocyto-genes* and *E. coli* (Figure 1). The release of potassium ions from the bacterial cells occurred immediately after the addition of *C. bonducella* seed oil at MIC concentra-tion following a sturdy loss along the specified time period (Figure 1a and 2b). However, no leakage of potassium ion was observed in control cells of *L. monocytogenes* and *E. coli* during the study.

### *Measurement of 260-nm materials*

Further, antibacterial mode of action of *C. bonducella* seed oil was visualized by the confirmation on the leakage of 260-nm absorbing materials when the test food-borne pathogens exposed to *C. bonducella* seed oil at MIC. In this assay, exposure of *L. monocytogenes* and *E. coli* to *C. bonducella* seed oil caused rapid loss of 260-nm absorbing materials from the bacte-rial cells. The optical density (260 nm) of the culture filtrates of *L. monocytogenes* and *E. coli* cells exposed to *C. bonducella* seed oil revealed an increasing release of 260-nm absorbing materials with respect to exposure time (Figure 2). However, no changes in the optical density of control cells of test pathogens were observed during the study. After 60 min of treatment, approxi-mately more than 2-fold increase was observed in the optical density of the bacterial cell culture filtrates treated with *C. bonducella* seed oil (Figure 2a and 2b).

### *Measurement of cell membrane permeability*

Figure 3 and 4 depict the mechanism for the effect of MIC exposure of *C. bonducella* seed oil on the membrane permeability of *L. monocytogenes* and *E. coli* in terms of their relative electrical conductivities. The *C. bonducella* seed oil expressed time-dependent effect in this assay, and the relative electrical conductivity of each test pathogen was increased timely. However, *C. bonducella* seed oil exerted significantly ($p<0.05$) higher effect on *L. monocytogenes* (Figure 3a) with increased proportion of relative electrical conductivity as compared to *E. coli* (Figure 3b). No relative electrical conductivity was

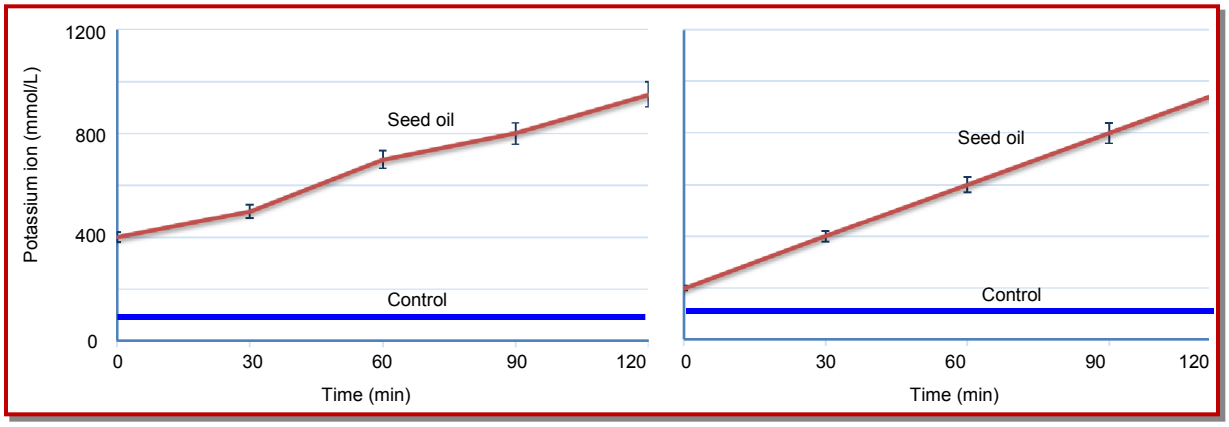


Figure 1: Effect of the *C. bonducella* seed oil on the leakage of potassium ions from the tested food-borne pathogenic bacteria *L. monocytogenes* (a) and *E. coli* (b)

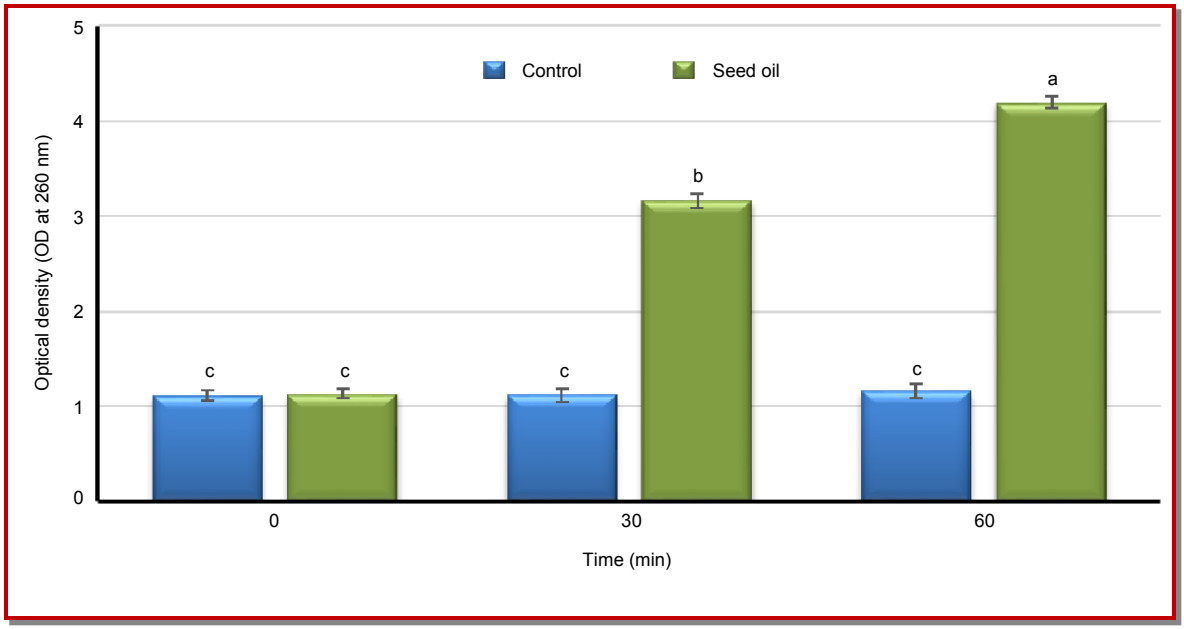


Figure 2: Effect of the *Caesalpinia bonducella* seed oil (CBSO) on the release rate of 260-nm absorbing material from *L. monocytogenes* NCIM 24563 (a) and *E. coli* ATCC 25922 (b). Data are expressed as mean ± SD (n = 3). Values with different superscript (a, b, c) are significantly different ($p<0.05$) between control and treatment groups

observed in untreated samples as a control.

## Discussion

In this study, membrane permeability parameters such as determination of extracellular ATP concentration, leakage of potassium ions, loss of 260 nm-absorbing materials, and measurement of relative electrical conductivity were used to determine the mode of action of *C. bonducella* seed oil against selected food-borne pathogenic bacteria. It was confirmed that *C. bonducella* seed oil had detrimental effect on damaging cell membrane integrity, resulting in the increased extracellular ATP concentration from the treated cells. The results of our study on the determination of extracellular ATP concentration showed an increasing rate of extracellular ATP concentrations after *L. monocytogenes* NCIM 24563 and *E. coli* ATCC 25922 cells exposed to *C. bonducella* seed oil.

This phenomenon may lead to significant impairment in membrane permeability of the tested bacteria by *C. bonducella* seed oil, which caused the intracellular ATP leakage through defective cell membrane as also reported previously (Herranz et al., 2001). It has been found that cells of *B. subtilis* treated with essential oil components resulted in the release of increased level of extracellular ATP pool (Helander et al., 1998).

The use of ATP has been confirmed in various cell functions including transport work moving substances across cell membranes which might be an important parameter to understand the mode of action of antibacterial agents. Subsequently, the antibacterial effect might be established because of proton motive force inhibition, mitochondrial respiratory inhibition, inhibition of substrate oxidation and active transportation, and loss of pool metabolites, as well as disruption of synthesis of macromolecules, proteins, lipids and polysaccharides may also occur. In general, leakage of intracellular material is induced by many antimicrobial agents resulting in the death of a cell (Denyer, 1990; Farag et al., 1989).

Moreover, in this study, *C. bonducella* oil exerted remarkable efficacy on the release of potassium ions from the treated cells of *L. monocytogenes* NCIM 24563 and *E. coli* ATCC 25922 cells at MIC concentration.

Previously Bajpai et al. (2013) also observed the effect of *C. cuspidata* oil on the cell membrane of target pathogens confirming that oil had significant effect on the release of potassium ions from the bacterial cells exposed to *C. cuspidata* oil.

The bacterial plasma membrane provides a permeability barrier to the passage of small ions such as potassium ions which are necessary electrolytes, facilitating cell membrane functions and maintaining proper enzyme activity. This impermeability to small ions is regulated by the structural and chemical composition of the membrane itself. Increases in the leakage of potassium ions will be an indication of disruption of this permeability barrier. Maintaining ion homeostasis is integral to the maintenance of the energy status of the cell including solute transportation, regulation of metabolism, control of turgor pressure and motility (Cox et al., 2001). Therefore, even relatively slight changes to

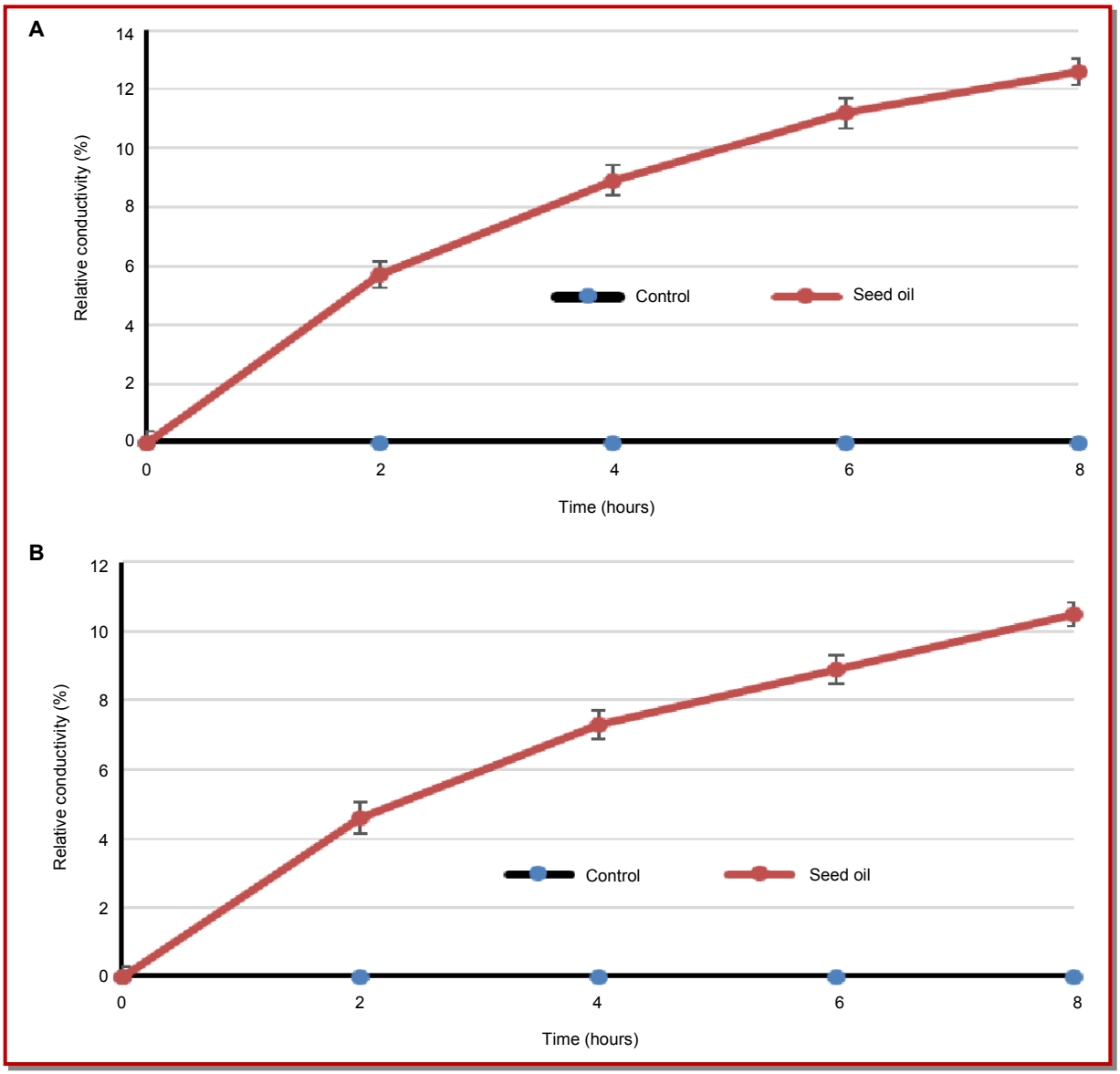


Figure 3: Effect of *Caesalpinia bonducella* seed oil on membrane permeability of *L. monocytogenes* NCIM 24563 (A) and *E. coli* ATCC 25922 (B). Data are expressed as mean ± SD (n = 3)

the structural integrity of cell membranes can severely affect cell metabolism and lead to cell death, and potassium ion efflux (Cox et al., 2001).

Measurement of specific cell leakage markers such as 260-nm-absorbing materials is an indication of membrane sensitivity to specific antimicrobial agent in relationship to control cells. In this study, oil of *C. bonducella* seeds exhibited significant potential on the release of 260 nm materials (DNA and RNA) from the cells of tested bacteria when exposed to MIC concentration. This directly indicates the confirmation of leakage of 260-nm absorbing materials from the bacterial cells treated with *C. bonducella* seed oil.

The effect of carvacrol, an essential oil component, on bacterial proton motive force was strongly correlated to the leakage of various substances, such as ions, ATP, nucleic acids (260 nm materials) and amino acids (Herranz et al., 2001).

The observation that the amount of loss of 260-nm-absorbing materials was as extensive as the leakage of potassium ions might indicate that the membrane structural damage sustained by the bacterial cells resulted in release of macromolecular cytosolic constituents (Farag et al., 1989). This suggested that monitoring $K^+$ efflux and release of 260-nm absorbing materials from *L. monocytogenes* NCIM 24563 and *E. coli* ATCC 25922 might be more sensitive indicators of membrane damage and loss of membrane integrity (Cox et al., 2001).

Furthermore, this study revealed that *C. bonducella* oil

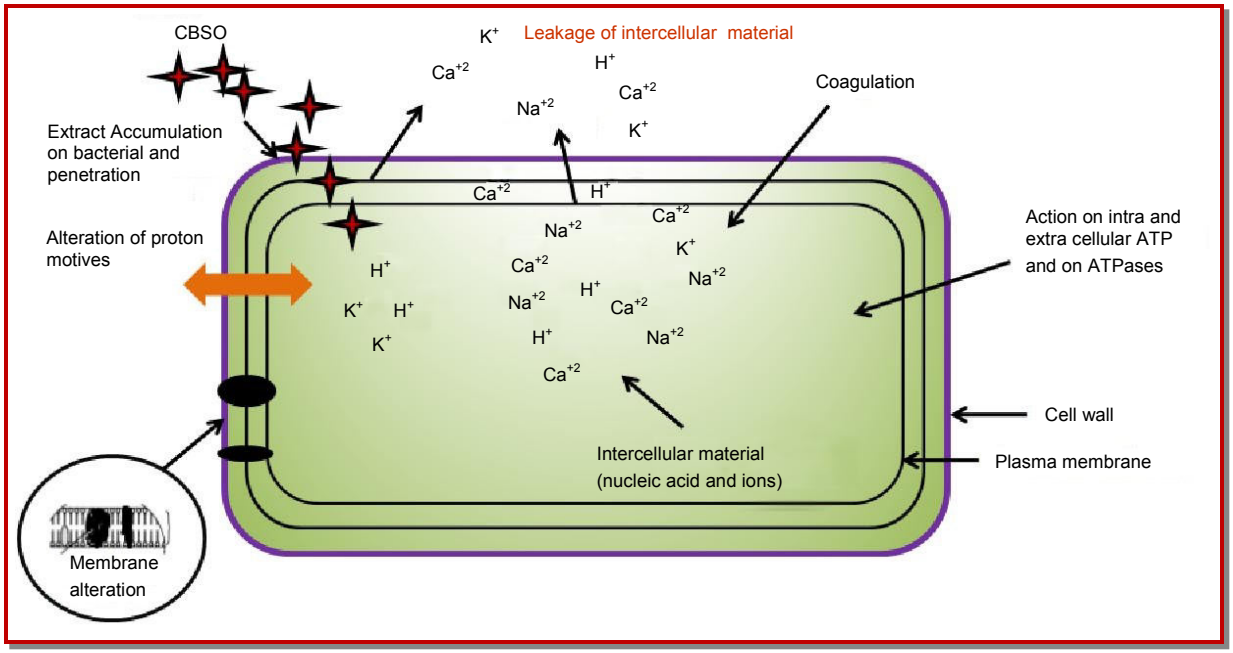


Figure 4: Proposed mechanism of the antibacterial mode of action of *Caesalpinia bonducella* seed oil against food-borne pathogenic bacteria

has ability to disrupt the plasma membrane as confirmed by the changes observed in the relative electrical conductivity of both the tested bacteria.

Similarly oil from different origins have also shown the remarkable effect on relative electrical conductivity parameters of bacterial pathogens (Patra et al., 2015).

Maintaining membrane permeable integrity is essential for overall metabolism of a bacterial cell hence, changes in the relative electrical conductivity on membrane integrity may severely hamper the cell metabolism, which may eventually lead to cell die (Cox et al., 2001). Based on the findings of this study it can be hypothesized that the accumulation of the oil in the plasma membrane caused instant loss of the cell integrity and became increasingly more permeable to ions and other essential metabolites that might be the reason for the establishment of the antibacterial activity of *C. bonducella* seed oil. Excessive leakage of cytoplasmic material is used as indication of gross and irretrievable damage to the plasma membrane (Cox et al., 1998).

Based on these facts and outcome of this study, a proposed mechanism of action of the antibacterial effect of *C. bonducella* seed oil against food-borne pathogenic bacteria is demonstrated in Figure 4.

These activities might be attributed to the presence of several biologically active components present in the seed oil of *C. bonducella* such as saponins, terpenoids, phenolics, flavonoids and polysaccharides (Ashebir and Ashenafi, 1999), as also supported by other researchers (Al-Reza et al., 2010; Rahman and Kang, 2010). In addition to this, the other major components of the *C. bonducella* seed oil which are well known for their antimicrobial efficacy such as bonducin, caesalpin, lysine, aspartic acid, stearic acid, tocopherol, campesterol, beta-sitosterol, stigmasterol, and avenasterol can contribute to potential antibacterial activity of seed oil of *C. bonducella* against the tested food-borne pathogens (Sultana et al., 2012).

The results of this study confirm that *C. bonducella* seed oil disrupted membrane functions of test food-borne pathogens. *C. bonducella* seed oil exerted its inhibitory effect through membrane permeabilization associated with membrane-disrupting effects leading to simultaneous reduction in cell viability, loss of 260-nm absorbing materials, leakage of potassium ions, release of ATP and increase in relative electrical conductivity, which confirmed the loss of membrane integrity. Based on these findings, it is concluded that *C. bonducella* seed oil showing significant antibacterial activity, can be used as a natural antimicrobial agent in food and pharmaceutical industries to control the growth of food -borne pathogens.

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**Author Info**
Shruti Shukla (Principal contact)
e-mail: shruti.shukla15@yahoo.com

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